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ORIGINAL ARTICLE

Involvement of Ascorbic Acid on Pea (*Pisum sativum* L.) seedlings exposed to Lead toxicity

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ABSTRACT
Lead (Pb) is an important environmental pollutant extremely toxic to plants. To assess Pb phytotoxicity, a hydroponic experiment was carried out using Pisum sativum L. on plant growth, chlorophyll pigments, MDA content, total protein and ascorbate peroxidase enzyme using biochemical, histochemical methods. Ascorbic acid (AA) is one of the most effective growth regulators against heavy metal stresses and acts as an antioxidant. Experiment showed that higher the concentration of heavy metals the more toxic effect to Pisum sativum L. The adverse effects of pb toxicity treatments on root and shoot were alleviated by the treatment of test plants with ascorbic acid.
Keywords: *antioxidant, hydroponic, lead, protein, treatment*

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INTRODUCTION

Peas (*Pisum sativum* L.), a cool-season crop grown for human consumption, Rotational crop, animal feed, or green manure and cover crop. Peas are a nutritious legume, containing 15 to 35% protein, and high concentrations of the essential amino acids lysine and tryptophan [1]. Apart from the natural weathering processes, Pb contamination of the environment has resulted in mining and smelting activities, Pb containing paints, gasoline and explosives as well as from the disposal of municipal sewage sludge's enriched in Pb, fuel combustion, synthetic fertilizers, and various industrial processes: building construction, Pb-acid batteries, bullets and shot, solder, pewter, and fusible alloys [2, 3]. Significant increases in the Pb content of cultivated soils has been observed near industrial areas [4]. Soils contaminated with Pb cause significant decreases in crop productivity thereby posing a serious problem for agriculture [5]. Pb is a pollutant that accumulates in soils, sediments, and water and is extremely persistent in the environment. Pb is not an essential element and it is toxic to living organisms. The primary effect of Pb toxicity in plants is a rapid inhibition of root growth, probably due to the inhibition of cell division in the root tip [5]. It was demonstrated that Pb caused inhibition of cell division in *Lemnaminor* roots. In several plants pieces, including *Triticum aestivum* [7], *Pisum sativum* [8], and *Sedum alfredii* [9], a decrease in the length and in root dry mass under Pb toxicity have been reported. It has been reported that Pb leads to the over production of reactive oxygen species (ROS) such as superoxide radicals (radical O^{2-}) and hydrogen peroxide (H_2O_2) in plant cells [11]. These can cause lipid peroxidation, membrane damages, and oxidative stress [10]. When pea (*Pisum sativum*) roots were exposed to 0.1 and 0.5mM of $Pb(NO_3)_2$, a rapid increase in superoxide anion (O^{-2}) and H_2O_2 levels occurs after 2 and 8h of Pb treatment, respectively [8]. They demonstrated that treatment with glutathione, a powerful antioxidant, decreased Pb^{2+} induced root cells death and reduced MAP kinases activity. An increase in H_2O_2 content upon Pb exposure was observed in response to Pb^{2+}, with an increase in CAT activity in *Triticum aestivum* [7], *Elsholtzia argyi*, and *Pisum sativum* [8]. Vitamin C (L-ascorbic acid) (AA) is the best well known compound used for antioxidant and markedly improve the inhibitory effects of oxidative stresses on plant growth and metabolism. AA is a small antioxidant molecule, water soluble and acts as a primary substrate in the cyclic pathway for enzymatic detoxification of a number of reactive oxygen species (ROS) such as

H_2O_2, and many other, harmful to normal functioning of plant metabolism. In addition, it acts directly to neutralize superoxide radicals (O^{2-}), singlet oxygen (O^{-}) or hydroxyl radical (OH^{-}) simply by acting as a secondary antioxidant during reductive recycling of the oxidized form of α-tocopherol [12]. The role of exogenous application of ascorbic acid on activities of antioxidant enzymes, growth is imperative to study. Although identified as a high yielding crop in many countries, *Pisum sativum L.* is very sensitive to environmental wavering and its responses to toxicants such as Pb need to be explored. Therefore, the main objective of this study was undertaken to examine the effect of ascorbic acid as antioxidant on growth,photosynthetic pigments, malondealdehide, protein and ascorbate peroxidase in order to obtained high quality yield in peas exposed to Pb stress.

MATERIALS AND METHODS

Plant growth and treatment with Pb and AA

Pisum sativum L cv. Line L_{12} seeds were sterilized with 5% sodium hypochlorite for 15 minute and washed extensively with distilled water, then germinated on moist filter paper in the dark at 27°C for one week. Then, seedlings of uniform size were transferred to plastic pots filled with perlite (5 plants per pot) and irrigated by distilled water for 14 days. The seedlings were then nourished with half-strange Hoagland solution for 6 days, during 3 leaves stage irrigation was continued with half-strange Hoagland solutions containing different concentrations of $Pb(NO_3)_2$ (0.25, 0.5, 1 mM) and ascorbic acid(0 and 1 mM) alone and together for 10 days. Plants were grown in a growth chamber at 25/22°C day/night temperatures and a 16-h/8-h light/dark photoperiod, with relative humidity between 60% and 70%. For the estimation of plant dry weight (DW), the plants were dried at 80°C for 48 h, to give a constant weight.

Growth Parameters

The plants were carefully uprooted from pots and washed thoroughly with running tap water. Plant growth was determined by measuring the length of the root and shoot system.

Determination of Photosynthetic pigments

Fresh leaf material (1.0 g) collected for each treatment was chopped into 0.5 cm segments and later extracted in 10 mL acetone (80%) at 4°C over-night for the estimation of chlorophyll (Chl) and carotenoid (Car) contents according to the methods of Arnon [13]. Following formulae were used to calculate Chl_a, Chl_b, total Chl and CAR contents after measuring the absorbance of supernatant on a spectrophotometer at 645, 663, and 480 nm.

Chla (mg g−1 FW) = [12.7 (OD663) −2.69 (OD645)] V/1000 x W

Chlb (mg g−1FW) = [22.9 (OD 645) −4.68 (OD 663)] V/1000 x W

Chlt (mg g−1FW) = [20.2 (OD 645) + 8.02 (OD 663)] V/100 x W

CAR (μg g−1 FW) = Acar/Emx100.

Where V is the volume of sample extract and W is the weight of the sample and A^{car} = (OD480) + 0.114 (OD663)−0.638 (OD645); E_{max}^{100} cm = 2500.

Estimation of malondialdehyde (MDA)

The level of lipid peroxidation was measured by estimating MDA, a decomposition product of peroxidized polyunsaturated fatty acid component of membrane lipid, using thiobarbituric (TBA) as the reactive material following the method of Heath and Packer (1968). The tissues were homogenized with 5% (w/v) TCA and 1mL of homogenate was mixed with 4 mL of TBA reagent (0.5% of TBA in 20% TCA). The reaction mixtures were heated at 95°C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 1900 g for 10 min. The absorbance of the coloured supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm and for absorbance at 532 nm of the correction blank. For the reference blank 1mL of 5 %(w/v) TCA was mixed whit 4mL TBA reagent and for the correction blank 1mL of tissue homogenate was mixed with 4mL of TBA reagent 20% (w/v) TCA. Concentration of MDA was calculated by using the extinction coefficient of 155 mM^{-1} cm^{-1} for MDA at 532 nm.

Determination of total protein

Proteins and metabolites were extracted from the leaf and root material. About 10 mg dry weight was used for extraction with 1 ml freshly prepared and pre-cooled extraction buffer ($MeOH:CHCl_3:H_2O$, 2.5:1:0.5). Samples were kept on ice for 10 min with regular agitation before centrifugation (4 min, 12,000*g*, 4 °C). The supernatant was mixed with 500 µl ultrapure water, shaken thoroughly and centrifuged (4 min, 12,000*g*, 4 °C). Measuring of total protein concentration performed by Bradford [14].

Ascorbate peroxidise

Ascorbate peroxidase activity was measured (Nakano and Asada, 1981) by a modified spectrophotometric method based on the rate of decrease in absorbance of ascorbate at 290 nm during ascorbate oxidation.

Statistical Analysis

All data were analyzed using the SPSS (Statistical Package for the Social Sciences) version 16.0. Data presented here are mean values and standard deviation (±SD). One way ANOVA was carried out using Post hoc multiple comparison from the Duncan's test at a significance level of $p < 0.05$.

RESULTS

Plant length

Effect of different concentrations of Pb (0.25, 0.5, 1 mM) and interaction with ascorbic acid (0 and 1 mM) on plant growth, expressed as the shoot length shown in Figure 1 and the root length in Figure 2. Pb exposure inhibited the growth of *Pisum sativum* L. significantly compared with control (Pb0, AA0), however, this inhibition was alleviated by the additions of 1mM AA.

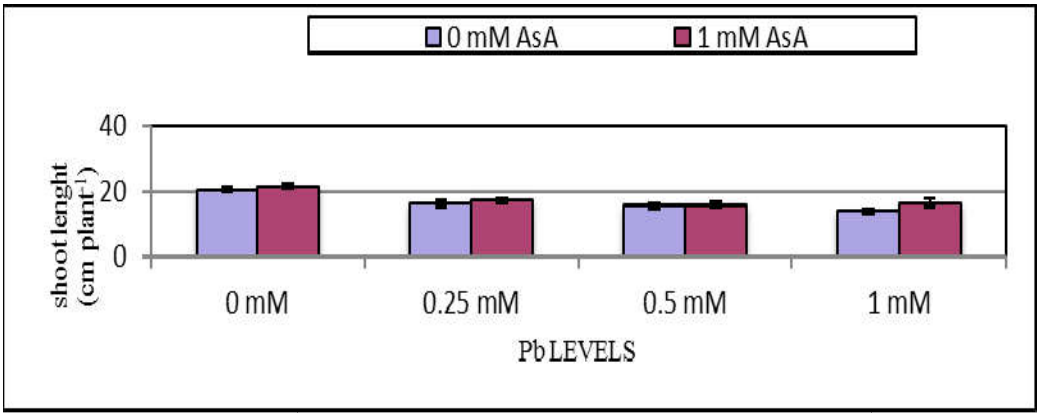

Figure 1. Effects of different concentrations of AA on shoot length (cm plant^{-1}) in *Pisum sativum* L. under Pb stress (X±S.E , n=4)((P<0.05).

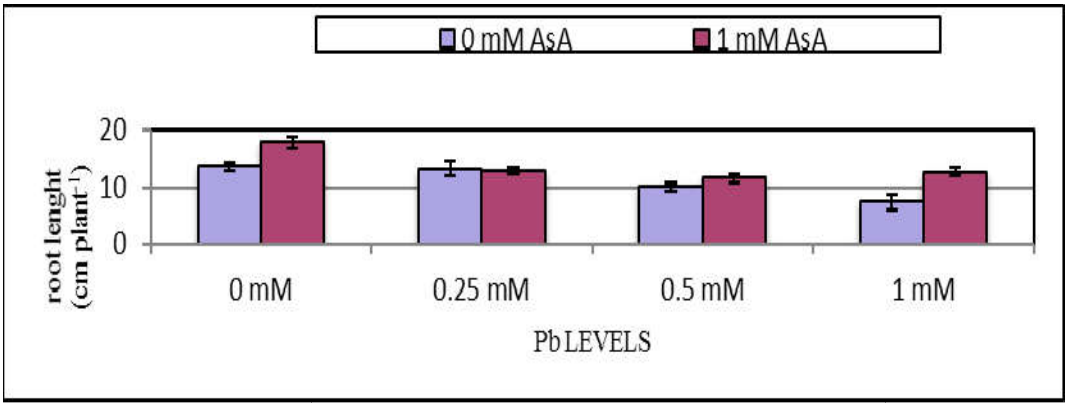

Figure 2. Effects of different concentrations of AA on root length (cm plant^{-1}) in *Pisum sativum* L. under Pb stress(X±S.E , n=4)((P<0.05).

Photosynthetic pigments (total chlorophyll, carotenoid)

A significant decrease in total chlorophyll contents was observed in the leaves of *Pisum sativum* L., which were exposed to Pb stress. Compared with Pb-stressed plants, the addition of 1 mM AA alleviated Pb toxicity in the photosynthetic apparatus (Figure 3). Maximum carotenoid was observed in the untreated leaf of *Pisum sativum* L. It increased significantly decrease with Pb treatment (Figure 4).

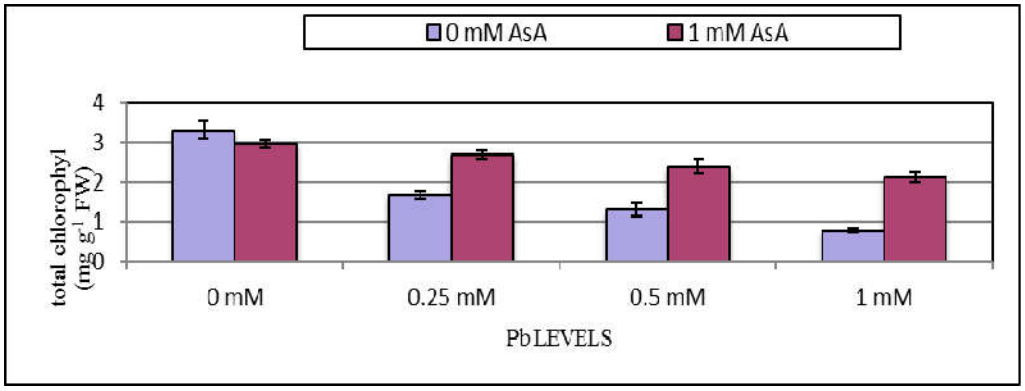

Figure 3. Effects of different concentrations of AA on total chlorophyll in leaf (mg g^{-1} FW) of *Pisum sativum* under pb stress (X±S.E , n=4)((P<0.05).

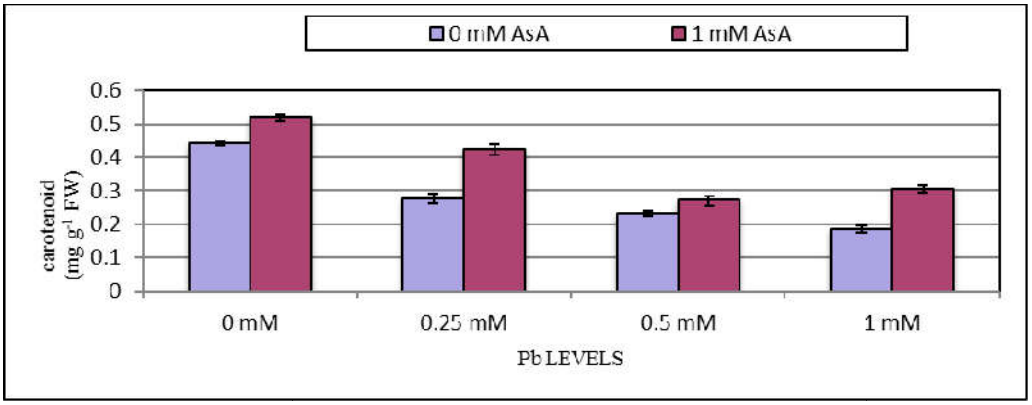

Figure 4. Effects of different concentrations of AA on carotenoid in leaf (mg g^{-1} FW) of *Pisum sativum* L. under Pb stress (X±S.E , n=4)((P<0.05).

Malondialdehyde content

In the present study, the concentration of MDA was increased (P<0.05) by Pb in metal-exposed plants. The application of AA to Pb treatments shows that the low concentration of AA (1 µM) can alleviate the effects of Pb stress on lipid peroxidation (Figure 5 and 6).

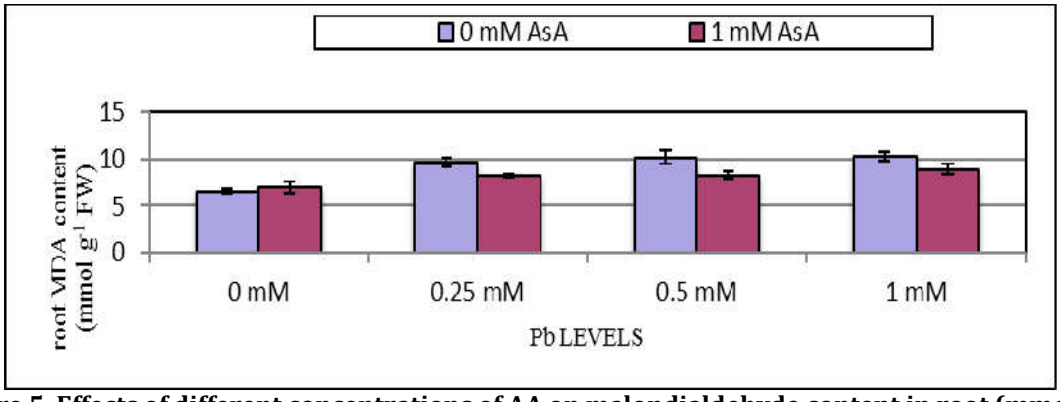

Figure 5. Effects of different concentrations of AA on malondialdehyde content in root (mmol g^{-1} FW) of Pisum *sativum* L. under Pb stress (X±S.E, n=4)((P<0.05).

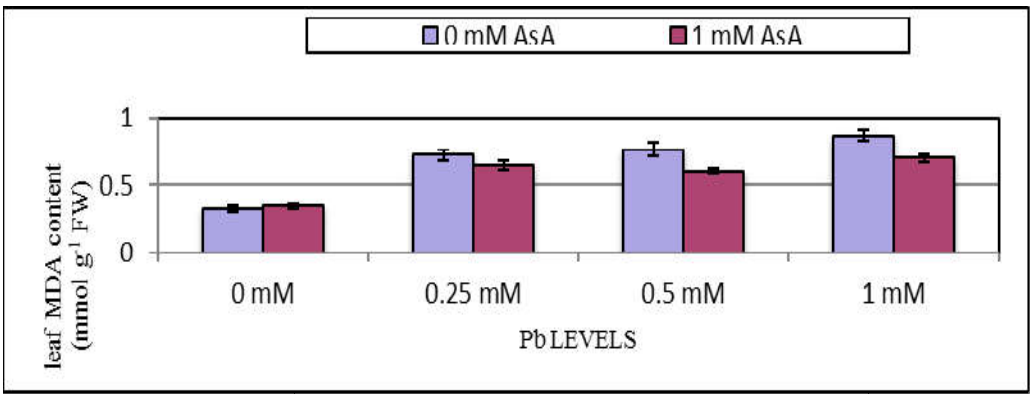

Figure 6. Effects of different concentrations of AA on malondialdehyde content in leaf (mmol g^{-1} FW) of *Pisum sativum* L. under Pb stress (X±S.E, n=4)((P<0.05).

Total protein

Pb concentration had adverse impact on protein of pea. The application of AA can alleviate the effects of Pb stress on total protein (Figure 7 and 8).

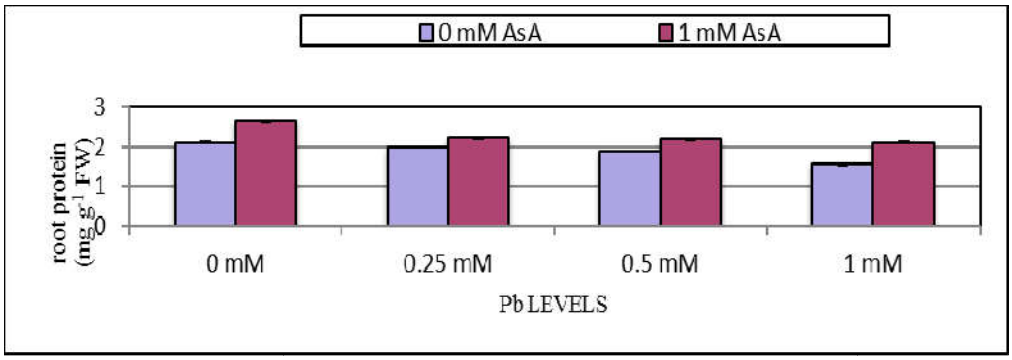

Figure 7. Effects of different concentrations of AA on protein in root (mg g^{-1} FW) of *Pisum sativum* L. under Pb stress (X±S.E, n=4)((P<0.05).

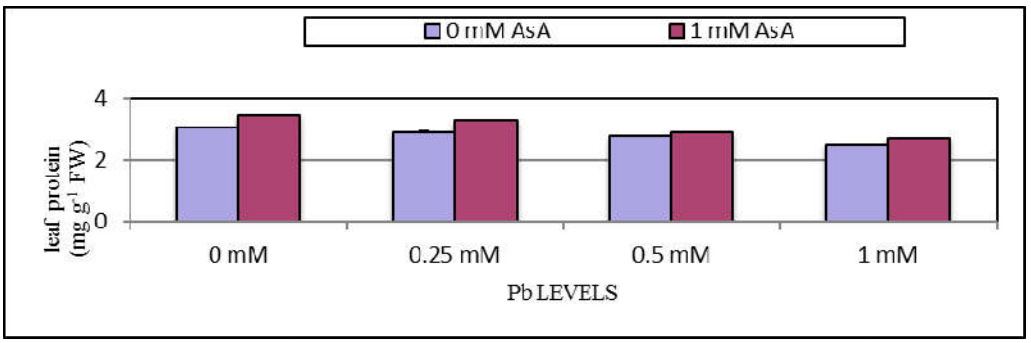

Figure 8. Effects of different concentrations of AA on protein in leaf (mg g^{-1} FW) of *Pisum sativum* L. under Pb stress (X±S.E, n=4)((P<0.05).

Ascorbate peroxidise

The activities of ascorbate peroxidase significantly higher in the plants treated with AA in comparison to the treated plant with Pb (Figure 9 and 10).

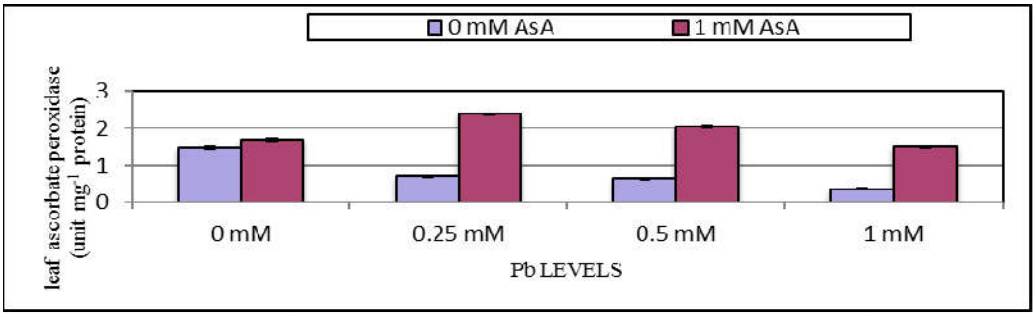

Figure 9. Effects of different concentrations of AA on ascorbate peroxidase in leaf (unit mg^{-1} protein) of *Pisum sativum* L. under Pb stress (X±S.E, n=4)((P<0.05).

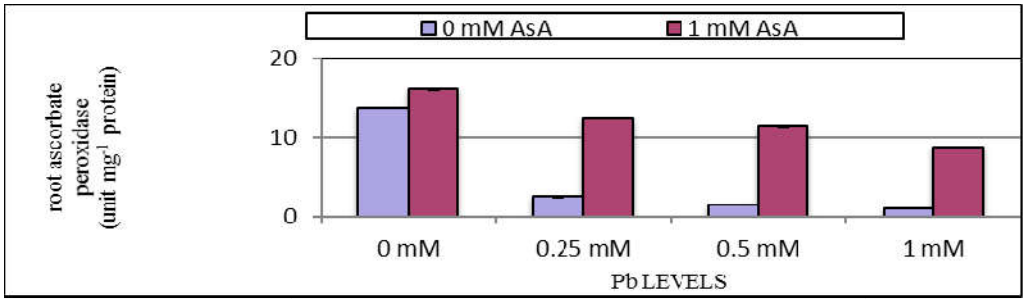

Figure 10. Effects of different concentrations of AA on ascorbate peroxidase in root (unit mg^{-1} protein) of *Pisum sativum* L. under Pb stress (X±S.E, n=4)((P<0.05).

DISCUSSION AND CONCLUSION

In this study the toxic effects of lead on the plant growth, total chlorophyll, carotenoid, MDA, total protein and ascorbate peroxidase were observed. To observe the beneficiary effect of ascorbic acid to ameliorate the lead toxicity 0 and 1Mm concentrations of ascorbic acid is needed to be utilized. From this study it can be concluded that the growth attributes increased significantly with the increasing number of days after the combined application of lead and ascorbic acid and ascorbic acid ameliorated the toxic effects of lead. The lowest plant height (17.37 cm) was observed in the presence of lead while highest value for the plant height (24.99 cm) was noticed after the application of ascorbic acid (table 1b). It has been reported that root and shoot growth was reduced in plants [15-17] by Pb-stress. The growth of legume plants grown on Pb ore tailings was reported to be drastically affected [18]. Similarly, in the present study, the root and shoot growth of pea seedling were inhibited by Pb-stress and the reduction was found to be concentration dependent. The reduction in root length and shoot length under Pb-stress may be due to the inhibition in cell elongation process [19]ne and Martin, 1977) or due to reduced mitotic activity as observed in lupin roots [20]. AA is as an ascorbate peroxidase substrate to scavenge H_2O_2 produced by ROS and plays important roles in plants under abiotic stress tolerance [21, 22]. AA affects the physiological activities of the plants. In this study application of AA increase root and shoot length of pea. It is now well understood that the endogenous level of AA will affect not only biosynthesis, but also that the levels and therefore, the signaling of these plant hormones play a tremendously significant role in the removal of a number of environmental stresses [23]. In this study, chlorophyll content showed minimum at 1 mM of Pb and increase with increase in AA treatment (table 1a). The chlorophyll content of the metal stressed leaves decreased with increased concentration of metal ion [24]. Application of AA increased markedly ($P \leq 0.05$) chlorophyll and carotenoid frequency under different Pb concentration here in tested (table 1a). The bioaccumulation of high Pb concentration can inhibit the chlorophyll synthesis, and then restrict the growth [25]. The exogenous application of AA might increase the absorption and translocation within the leaf in addition to enhancement of the biosynthesis of photosynthetic pigments and improvement the nutritional status of wheat [26, 27]. The hydroxyl radical is known to be the most potentially toxic species. Through the Haber-Weiss reaction H_2O_2 and $O_2^{\bullet -}$ can be transformed to the highly reactive oxidant $OH^{\bullet -}$, which causes lipid peroxidation in plant cell [28]. MDA is a cytotoxic product of lipid peroxidation and an indicator of membrane damage from oxidative stress. Pb toxicity is reported to induce the increase of MDA in *Talinum triangulare* [29]. As excess of heavy metals stimulate the formation of free radicals. In this study, increase levels of MDA with increasing Pb concentration were found (table 1a).

Protein concentrations decrease for leaves with 0.25 mM Pb then almost unchanged and a peak is observed for roots with 1mM Pb. This is possibly a result of the induction of stress proteins, which may comprise various antioxidant enzymes [30]. It could also result from the production of phytochelatins aimed to detoxify Pb ions [31]. The chemical and biological properties of L-ascorbic acid suggest that it can act as an antioxidant. When lead-stressed plants were treated with AA at the concentration of 1mM, the shoot protein contents were 3.74, 3.30, 2.91 and 2.72 mg/g, , in comparison with 3.5, 2.92, 2.80 and 2.5 mg/g ,respectively when treated with 0, 0.25, 0.5 and 1mM $Pb(NO_3)_2$ only (table 1a). Similar to this, it has been widely reported that the protein contents were increased after exogenous application of AA in potato [32] and chickpea [33].

Pb-stress caused a significant decrease in the ascorbate peroxidase enzyme activity in pea (table 1b). A considerable increase in the peroxidase activity of pea plants was observed with AA application (Figure 1b). ascorbate peroxidase activity of plants maintained at 1 mM $pb(NO_3)_2$ level was 0.36 mg/g, which slightly increased to 1.49 mg/g tissue after AA application. It has been reported that Pb leads to the over production of reactive oxygen species (ROS) such as super oxide radicals (radical O^{2-}) and hydrogen peroxide (H_2O_2) in plant cells [11]. These can cause lipid peroxidation, membrane damages, and oxidative stress [10]. When pea (*Pisum sativum* L.) roots were exposed to 0.1 and 0.5mM of $Pb(NO_3)_2$, a rapid increase in super oxide anion (O^{-2}) and H_2O_2 levels occurs after 2 and 8 h of Pb treatment, respectively [8]. These higher levels of antioxidant enzymes might be attributed to their property to help develop the plant's resistance against oxidative damage. Athar *et al.* [34] reported an increase in antioxidant enzyme activities in wheat plants after AA application.

We can conclude that adverse effects of pb stress on growth, photosynthetic pigments, MDA, total protein and ascorbate peroxidase enzyme of pea plants were significantly improved by exogenous application of AA. Hence higher concentration of any kind of heavy metal may affect the overall growth and development, and productivity of the plants. The AA might overcome the destructive effects of heavy metal by increasing the endogenous levels of antioxidant enzymes, which in turn was reflected in improved growth and the other reproductive characters.

Table 1a. Effects of different concentrations of AA on carotenoid, total Chlorophyll, Leaf and Root protein, Leaf and Root MDA content in *Pisum sativum* L. under Pb stress.

Pb (mM)	Ascobic Acid (mM)	Carotenoied (mg/g FW)	Total Chl (mg/g FW)	Root Protein (mg/g FW)	Leaf Protein (mg/g FW)	Leaf MDA content (nmol.g-1.FW)	Root MDA content (nmol.g-1.FW)
0	0	0.442±0.009 b	3.315±0.222 a	2.106±0.010 c	3.053±0.008 c	0.324±0.02 e	6.546031±0.18 d
	1	0.520±0.011 a	2.973±0.093 b	2.643±0.004 a	3.741±0.0004 a	0.349±0.02 e	6.928716±0.66 cd
0.25	0	0.277±0.013 cd	1.670±0.097 f	1.992±0.012 d	2.927±0.0014 d	0.727±0.04 bc	9.640963±0.49ab
	1	0.425±0.018 b	2.702±0.097 c	2.233±0.016 b	3.307±0.0006 b	0.653±0.03 cd	8.182777±0.15 bc
0.5	0	0.232±0.008 d	1.310±0.177 g	1.879±0.010 e	2.801±0.0016 f	0.771±0.05 b	10.18967±0.76 a
	1	0.275±0.019 cd	2.403±0.185 d	2.188±0.009 b	2.911±0.0004 e	0.609±0.02 d	8.304136±0.39 bc
1	0	0.185±0.0.010 e	0.872±0.042 h	1.553±0.018 f	2.500±0.0014 h	0.872±0.04 a	10.3321±0.46 a
	1	0.305±0.010 c	2.110±0.130 e	2.109±0.011 c	2.720±0.0011 g	0.707±0.03 bc	8.926477±0.53ab

Means followed by the same letter are not significantly different (P < 0.05)

Table 1b. Effects of different concentrations of AA on shoot and Root length, shoot and Root APX content in *Pisum sativum* L. under Pb stress.

Pb (mM)	Ascorbic Acid (mM)	Shoot Length (cm)	Root length (cm)	Leaf APX content (µmol.g-1.FW)	Root APX content (µmol.g-1.FW)
0	0	20.625±0.80 a	3.25±0.25 a	1.472±0.026 d	13.78±0.013 b
	1	21.625±0.94 a	3.375±0.47a	1.697±0.021 c	16.22±0.078 a
0.25	0	16.375±0.85 bc	3.375±0.24 a	0.690±0.019 e	2.473±0.025 f
	1	17.25±0.78 b	3.125±0.38 a	2.398±0.018 a	12.42±0.024 c
0.5	0	15.75±0.83 bc	3±0.35 a	0.627±0.023 e	1.420±0.014 g
	1	16±0.68 bc	3±0.54 a	0.047±0.025 b	11.45±0.036 d
1	0	14.125±0.72 a	3.25±0.52 a	0.362±0.023 f	0.972±0.008 h
	1	16.5±1.32 bc	3.125±0.43 a	1.495±0.022 d	8.725±0.010 e

Means followed by the same letter are not significantly different (P < 0.05)

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